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Competition for electron donors among nitrate reducers, ferric iron reducers, sulfate reducers, and methanogens in anoxic paddy soil

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Abstract Slurries of anoxic paddy soil were either freshly prepared or were partially depleted in endogenous electron donors by prolonged incubation under anaerobic conditions. Endogenous NO_3^- was reduced within 4 h, followed by reduction of Fe^{3+} and SO_4^{2-}, and later by production of CH_4. Addition of NO_3^- slightly inhibited the production of Fe^{2+} in the depleted but not in the fresh paddy soil. Inhibition was overcome by the addition of H_2, acetate, or a mixture of fatty acids (and other compounds), indicating that these compounds served as electron donors for the bacteria reducing NO_3^- and/or ferric iron. Addition on NO_3^- also inhibited the reduction of SO_4^{2-} in the depleted paddy soil. This inhibition was only overcome by H_2, but not by acetate or a mixture of compounds, indicating that H_2 was the predominant electron donor for the bacteria involved in NO_3^- and/or SO_4^{2-} reduction. SO_4^{2-} reduction was also inhibited by exogenous Fe^{3+}, but only in the depleted paddy soil. This inhibition was overcome by either H_2, acetate, or a mixture of compounds, suggesting that they served as electron donors for reduction of Fe^{3+} and/or SO_4^{2+}. CH_4 production was inhibited by NO_3^- both in depleted and in fresh paddy soil. Fe^{3+} and SO_4^{2-} also inhibited methanogenesis, but the inhibition was stronger in the depleted than in the fresh paddy soil. Inhibition of CH_4 production was paralleled by a decrease in the steady state concentration of H_2 to a level which provided a free enthalpy of less than $\Delta G = -17\ kJ\ mol^{-1}$ CH_4 compared to more than $\Delta G = -32\ kJ\ mol^{-1}$ CH_4 in the control. The results indicate that in the presence of exogenous Fe^{3+} or SO_4^{2+}, methanogenic bacteria were outcompeted for H_2 by bacteria reducing Fe^{3+} or SO_4^{2+}.

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Introduction

In paddy soil, degradation of organic matter is achieved by the sequential reduction of O_2, NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, and CO_2 (Ponamperuma 1972; Patrick and Reddy 1978). This sequence follows the thermodynamic theory that predicts that electron acceptors with a higher redox potential will be reduced first (Zehnder and Stumm 1988). All the reactions are catalyzed by microorganisms which use one of these compounds as an electron acceptor. One reason why the different electron acceptors are used sequentially is seen in the dominance of one type of microorganism in a given interval. This dominance is believed to be caused by the competition for common electron donors, which is won by those microorganisms that use the electron acceptor with the highest redox potential.

The competition concept was first presented for competition between sulfate reducers and methanogens in sediments of Lake Mendota (Winfrey and Zeikus 1977) and was later validated for various anoxic environments (King 1984; Ward and Winfrey 1985). The competition concept was then generalized to the competition between bacteria reducing Mn^{4+}, Fe^{3+}, and SO_4^{2-}, and producing CH_4 (Lovley and Goodwin 1988; Lovley 1991). The competition concept was first based on the observation that sulfate-reducers have a higher affinity (i.e., a lower K_m) for H_2 than methanogens (Kristjansson et al. 1982; Robinson and Tiedje 1984), and was later extended by the observation that SO_4^{2-}-reducers have a lower threshold for H_2 than methanogens (Lovley et al. 1982; Lovley 1985). That bacteria using electron acceptors with a higher redox potential also deplete electron donors to lower threshold concentrations has been validated in various defined bacterial cultures using H_2 as an electron donor in the presence of different electron acceptors (Cord-Ruwisch et al.

1988; Lovley et al. 1989; Seitz et al. 1990; Dolfing and Tiedje 1991).

There are also examples of anoxic environments where the operation of different redox reactions is not mutually exclusive (Mountfort et al. 1980; King 1984; Isa et al. 1986; Westermann and Ahring 1987; Qatibi et al. 1990; Lovley 1991) or cannot be explained satisfactorily by the competition concept (Conrad et al. 1987a). The basic operation of competition has not so far been rigorously tested in anoxic paddy soil.

Therefore, we investigated the interaction between the reduction of NO_3^-, Fe^{3+}, and SO_4^{2-} and the production of CH_4 in slurries of anoxic paddy soil which was either used in the fresh state or after prolonged incubation to deplete available electron donors.

Materials and methods

The paddy soil was obtained in 1991 from an Italian rice field located near Vercelli in the valley of the river Po. The site, soil characteristics, management, and seasonality have been described in earlier studies (Schütz et al. 1989; Rothfuss and Conrad 1993). The soil was stored in dry lumps at room temperature. It had a organic C content of 2.7%. Fresh slurries of anoxic paddy soil were prepared by suspending sieved (1 mm mesh) soil in demineralized water (1 g dry weight soil plus 1 ml H_2O). Anoxic paddy soil that was partially depleted in electron donors was prepared by incubating soil slurries for about 3 months at 30 °C under an N_2 atmosphere. Anoxic incubation was chosen to mimic the situation in a flooded rice field, although oxic incubation gives a more rapid depletion of organic matter (Mayer and Conrad 1990). Following incubation the soil was dried for about 1 week at 30 °C under air. The soil was then sieved (1 mm) and stored at room temperature. The drying may have caused a breakdown of resistant organic matter and an increase in electron donors (VanSchreven 1968), but was done to allow storage of the soil. This treatment resulted only in a partial depletion of the available organic matter, but experience has shown that rates of CH_4 production are decreased (Mayer and Conrad 1990). The partially depleted soil had an organic C content of 2.3%. For these experiments, the partially depleted soil was suspended in demineralized water (1 g dry weight soil plus 1 ml H_2O).

To measure the reduction of NO_3^-, Fe^{3+}, and SO_4^{2-}, the slurry (60 ml) was put together with a teflon-coated magnetic bar into a glass bottle (100 ml) with a glass tube blown to the neck and closed with a black rubber stopper. The headspace of the bottle was flushed with N_2 using the glass tube as the inlet and the loose rubber stopper as the outlet. After closing the bottle, it was incubated at 30 °C on a slowly rotating device. Samples (1.5 ml) of the slurry were taken with a pipette while simultaneously gassing the headspace with N_2 and stirring the slurry. The samples were centrifuged, filtered through regenerated cellulose membrane filters (0.45 µm), and stored frozen (−20 °C) until analysis of NO_3^- and SO_4^{2-} by ion chromatography (Bak et al. 1991). Other samples were taken and immediately analyzed for Fe^{2+} with the ferrozin reagent (Stookey 1970), using a slightly modified version of the technique described by Lovley and Phillips (1987). About 0.5 g of paddy soil slurry was transferred under anoxic conditions into a tube with 4.5 ml 0.5 *M* HCl. The exact amount of sample was determined gravimetrically. The sample was extracted by vortexing for 1 min. An aliquot (100 µl) of this mixture was mixed with 1 ml of ferrozin reagent (0.1% weight ferrozin in 50 m*M* HEPES buffer pH 7) and centrifuged for 2 min at 10000 **g**. The extinction of the supernatant was measured at 562 nm in a photometer. Total extractable Fe was analyzed after reduction to Fe^{2+} with hydroxylamine (Chao and Zhou 1983; Lovely and Phillips 1987). After extraction of the soil slurry with 0.5 *M* HCl as described above, an aliquot (100 µl) was mixed with 2 ml of a solution of 0.25 *M* hydroxylamine hydrochloride in 0.25 *M* HCl and incubated for 2 h at 60 °C. Then, 100 µl of the mixture was mixed with 1 ml ferrozin reagent and processed as described above. The concentration of extractable Fe^{3+} was determined by the difference between total extractable Fe and Fe^{2+}. To measure CH_4 production, the slurry (45 ml) was poured into serum bottles (120 ml) which were flushed with N_2, closed with black rubber stoppers, and incubated at 30 °C without agitation. Gas samples were taken from the headspace after the bottles had been heavily shaken by hand, and analyzed for H_2, CO_2, and CH_4 as described previously (Conrad et al. 1987b).

In some experiments, the soil slurries were amended with a suspension of Fe^{3+}, which was prepared by precipitation with alkali (Schwertmann and Cornell 1991), using 1 liter of 0.5 *M* $FeCl_3$ solution (pH 1–2) neutralized with 5 *M* NaOH under rapid stirring. The samples were then left overnight to allow the Fe^{3+} crystals to settle. The clear supernatant was sucked off, and the precipitate dialyzed for 3 days against H_2O which was repeatedly exchanged (about every 8 h). The resulting suspension of Fe^{3+} was then free of electrolytes, and was stored in a 1 *M* suspension at room temperature.

The standard Gibbs free energy at pH 7 ($\Delta G'_0 = -135.6$ kJ mol^{-1} CH_4) for the H_2/CO_2-dependent production of CH_4 was from Thauer et al. (1977). The Gibbs free energies (ΔG) under the actual incubation conditions were determined for the H_2/CO_2-dependent production of CH_4 using the concentrations of the reactants and products which were actually measured and inserting them into the Nernst equation as described by Conrad et al. (1986). The pH was 7.3, and the bicarbonate concentration was 25 and 20 m*M* in fresh and depleted soil, respectively. The partial pressures of H_2 and CH_4 are given in the results.

Results

The sequential operation of redox processes in fresh paddy soil slurry incubated under anoxic conditions is shown in Fig. 1a. The endogenous concentration of NO_3^- (about 200 µ*M*) was reduced within 4 h to undetectable concentrations (< 0.1 µ*M*). Accumulation of Fe^{2+} started almost immediately. In a separate experiment, Fe^{3+} was measured as the difference between Fe^{2+} and total extractable Fe. The initial concentration of Fe^{3+} was 110 µmol g^{-1} dry weight soil. Fe^{3+} decreased in parallel with the accumulation of Fe^{2+}. After 8 days of incubation, when Fe^{3+} had reached a concentration of about 40 µmol g^{-1} dry weight, it stopped decreasing and Fe^{2+} no longer accumulated, indicating that the residual Fe^{3+} was not being further reduced. Concentrations of SO_4^{2-} increased during the first day and then progressively decreased until a threshold concentration of about 1–2 µ*M* was reached after 10 days (Fig. 1a). Moreover, the reduction of Fe^{3+} and of SO_4^{2-} occurred simultaneously. Production of CH_4 started after 6 days and accelerated until the full rate was reached after 10 days of incubation. The rates of the different reactions observed in this and in similar experiments are summarized in Table 1. The experiment was also conducted with paddy soil which had been partially depleted for endogenous electron donors by prolonged incubation under anoxic conditions followed by drying (Fig. 1b). The different redox processes showed a similar sequential pattern to that observed with fresh paddy soil The main differences were the lack of NO_3^- in the beginning and the lower rate of CH_4 production in the end (Table 1).

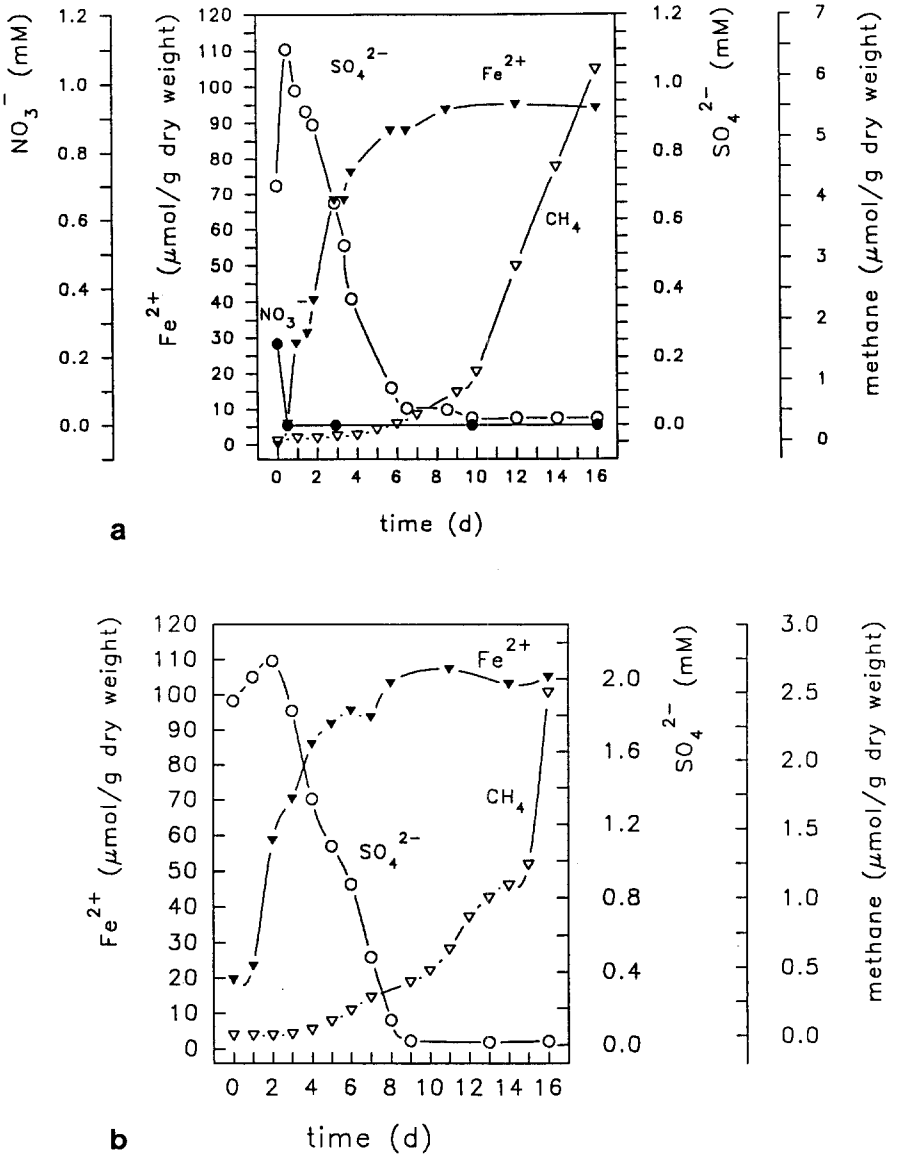


Fig. 1 Sequential operation of redox processes in fresh (**a**) and depleted (**b**) anoxic paddy soil slurry, showing reduction of NO_3^- and SO_4^{2-} and production of Fe^{2+} and CH_4

In depleted anoxic paddy soil, no endogenous NO_3^- was present. Added NO_3^- was rapidly consumed within 12 h. In order to study the effect of NO_3^- on the reduction of Fe^{3+}, NO_3^- was repeatedly added to the anoxic paddy soil (Fig. 2). After 24 h NO_3^- was generally no longer detectable. There was no accumulation of NO_2^-. The repeated addition of NO_3^- did not affect the accumulation of Fe^{2+} by reduction of endogenous Fe^{3+} in fresh paddy soil (Fig. 2a), and resulted only in a slight, almost insignificant inhibition in depleted paddy soil (Fig. 2b). This slight inhibition was completely relieved when the paddy soil was simultaneously amended with an electron donor in the form of H_2, acetate, or a mixture of fatty acids (butyrate, propionate, glucose, ethanol and fumarate; Fig. 2c). H_2 seemed to stimulate Fe^{2+} production, indicating the presence of H_2-using ferric iron reducers.

A similar experimental protocol was used to study the effect of NO_3^- on SO_4^{2-} reduction (Fig. 3). Again, NO_3^- had no effect in the fresh paddy soil (Fig. 3a), but resulted in complete inhibition of SO_4^{2-} reduction in the paddy soil which had been depleted in endogenous electron donors (Fig. 3b). This inhibition was completely relieved by H_2, but not by acetate or a mixture of fatty acids and other electron donors (Fig. 3c).

Reduction of SO_4^{2-} was also inhibited by the addition of Fe^{3+} but only, however, in the depleted and not in the fresh paddy soil (Fig. 4a, b). Immediately after the addi-

Table 1 Rates of microbial redox processes in fresh and depleted paddy soil (mean ± SD) of triplicates

Reaction	Rate (µmol g^{-1} dry weight day^{-1})	
	Fresh soil	Depleted soil
NO_3^--reduction	0.84 ± 0.09	0.85[a]
Fe^{2+} production	14.00 ± 1.60	16.40 ± 1.80
SO_4^{2-} reduction	0.20 ± 0.03	0.25 ± 0.04
CH_4 production	1.20 ± 0.21	0.57 ± 0.16

[a] Measured after addition of NO_3^-

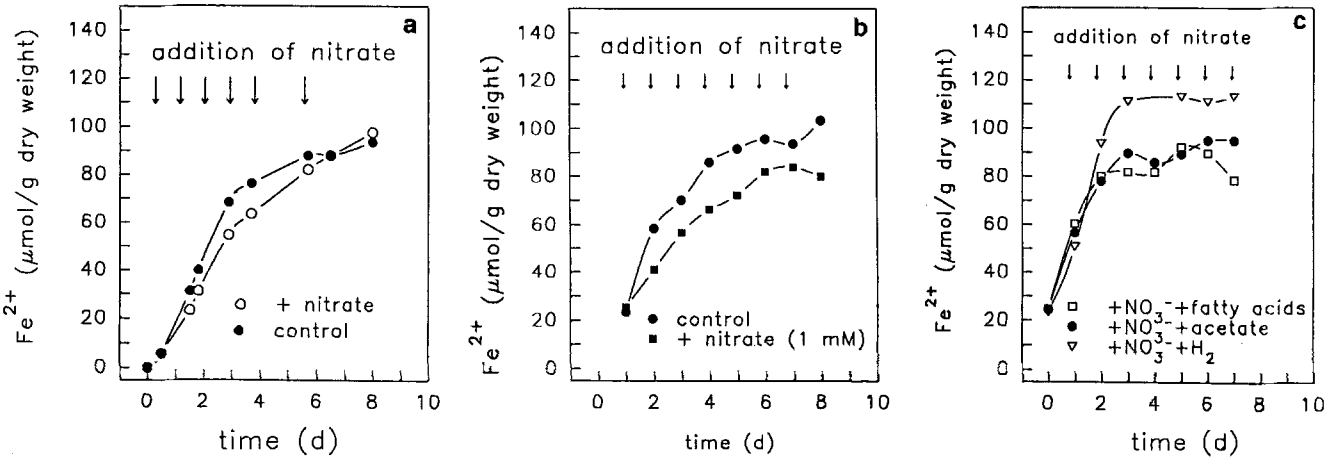


Fig. 2 Effect of repeated additions (*arrows*) of NO_3^- (1 m*M*) on production of Fe^{2+} in fresh (**a**) or depleted (**b**) anoxic paddy soil, and effect of simultaneous additions (each time NO_3^- was added) of acetate (2 m*M*), fatty acids plus other compounds (200 µ*M* each of butyrate, propionate, glucose, ethanol, and fumarate), or H_2 (100%) as electron donors to the depleted paddy soil (**c**)

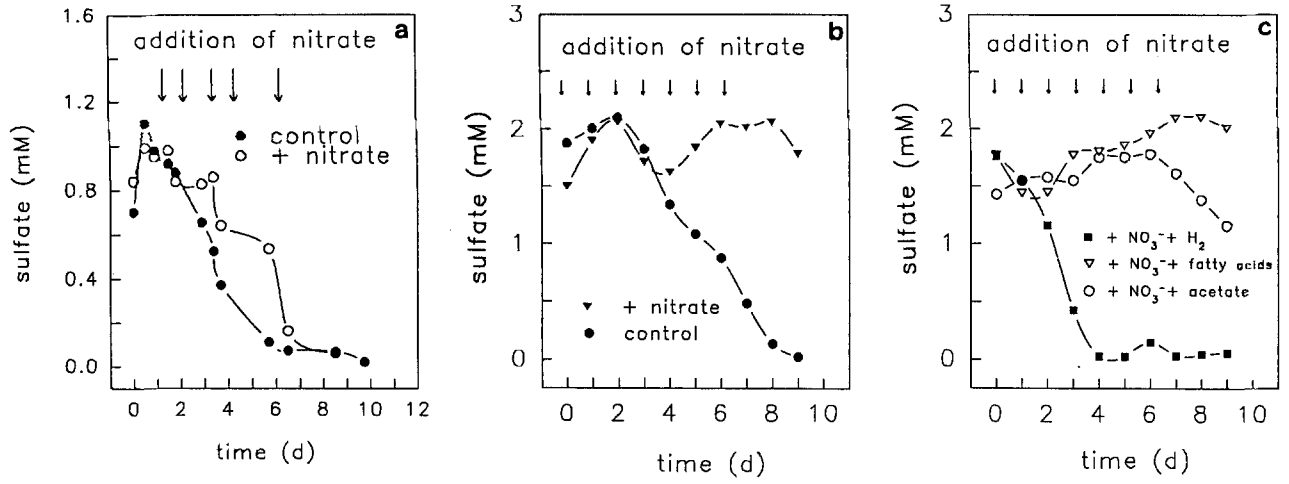


Fig. 3 Effect of repeated additions (*arrows*) of NO_3^- (1 m*M*) on reduction of SO_4^{2-} in fresh (**a**) or depleted (**b**) anoxic paddy soil, and effect of simultaneous additions (each time NO_3^- was added) of acetate (2 m*M*), fatty acids plus other compounds (200 µ*M* each of butyrate, propionate, glucose, ethanol and fumarate), or H_2 (100%) as electron donors to the depleted paddy soil (**c**)

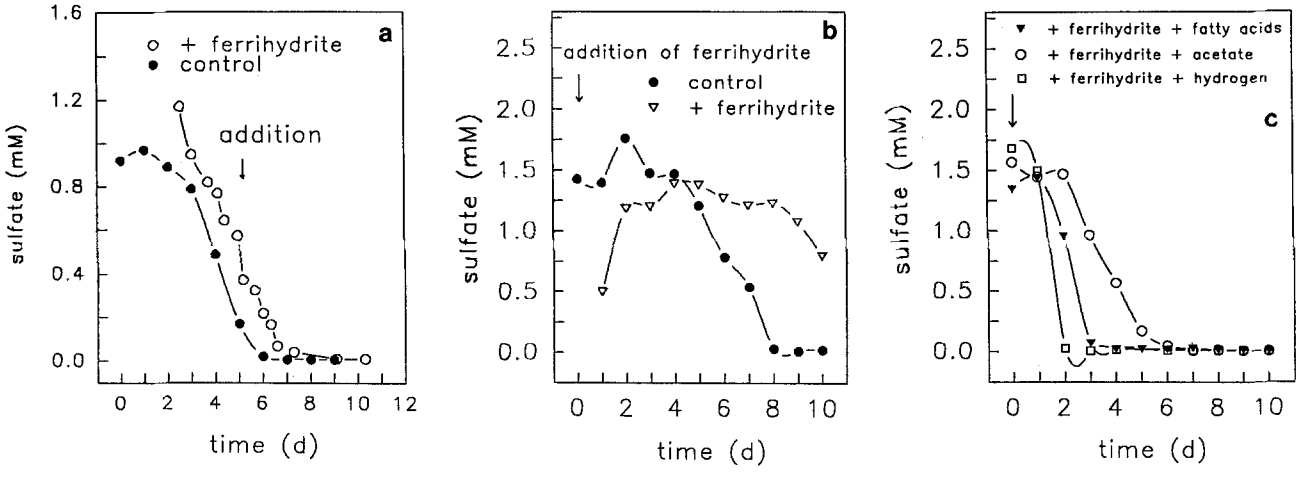


Fig. 4 Effect of addition of Fe^{3+} (30 m*M*) on reduction of SO_4^{2-} in fresh (**a**) or depleted (**b**) anoxic paddy soil, and effect of simultaneous addition of acetate (6 m*M*), fatty acids plus other compounds (200 µ*M* each of butyrate, propionate, glucose, ethanol and fumarate), or H_2 (100%) as electron donors to the depleted paddy soil (**c**)

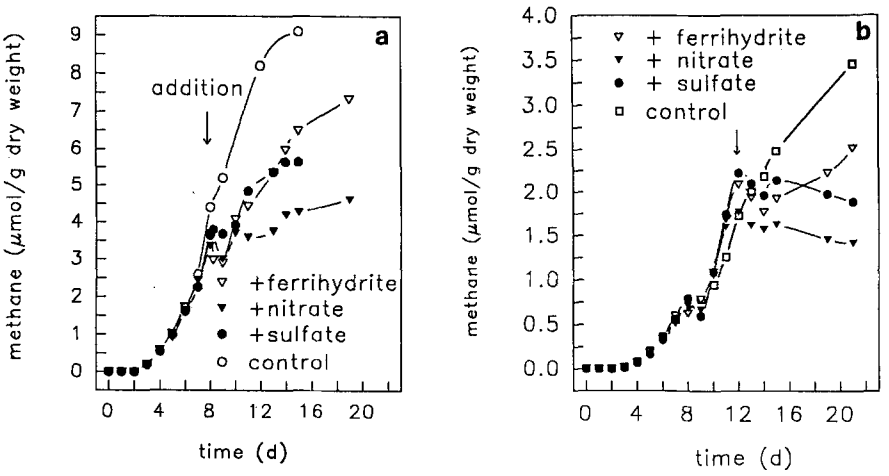


Fig. 5 Effect of the addition of Fe^{3+} (40 m*M*), SO_4^{2-} (15 m*M*), or NO_3^- (10 m*M*) on the production of CH_4 in fresh (**a**) or depleted (**b**) anoxic paddy soil

tion of Fe^{3+}, the concentration of SO_4^{2-} decreased, but recovered 1 day later (Fig. 4b). This effect was reproducible and was possibly due to adsorption effects between SO_4^{2-} and Fe^{3+} (Ponnamperuma 1972), but was not further investigated. The inhibition of SO_4^{2-} reduction by Fe^{3+} was completely relieved by the addition of exogenous electron donors such as H_2, acetate, or a mixture of fatty acids with other compounds (Fig. 4c).

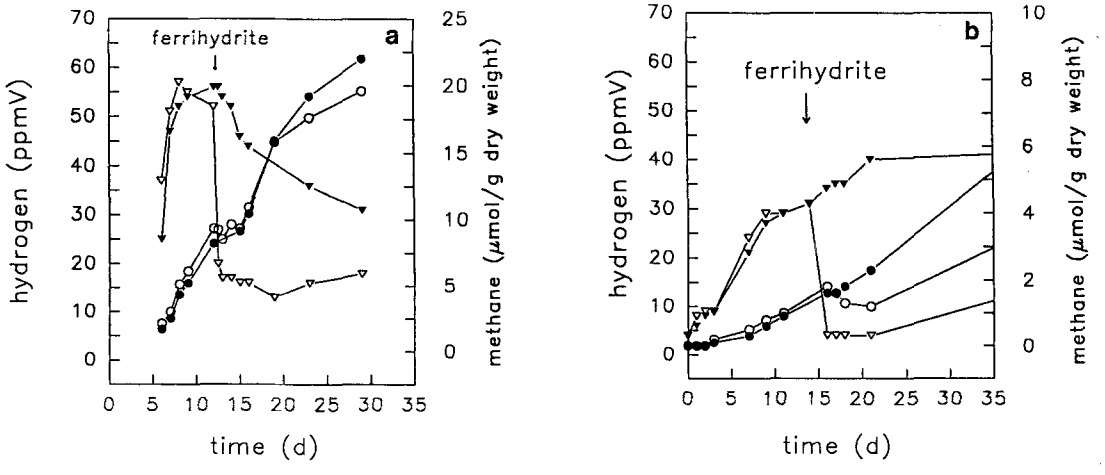
The production of CH_4 was inhibited by any of the other electron acceptors, i.e., by the addition of SO_4^{2-}, Fe^{3+}, or NO_3^- (Fig. 5). The addition of NO_3^- generally resulted in complete inhibition of CH_4 production. The addition of SO_4^{2-} resulted in complete inhibition in the depleted (Fig. 5) but only in partial inhibition in the fresh (Fig. 5a) paddy soil. The addition of Fe^{3+} generally resulted only in a partial inhibition of CH_4 production (Fig. 5).

Inhibition of CH_4 production by Fe^{3+} and SO_4^{2-} was paralleled by a decrease in the H_2 partial pressure until a new steady state was reached at a lower H_2 partial pressure (Figs. 6, 7). In fresh soil, however, H_2 did not decrease to such low concentrations as in depleted soil. The concentrations were used to calculate the Gibbs free energies available from H_2-dependent CH_4 production before and after addition of the exogenous electron acceptor (Table 2). The results indicate that CH_4 produc-

Table 2 Effect of SO_4^{2-} and Fe^{3+} on the Gibbs free energies of H_2-dependent methanongenesis in fresh and electron donor-depleted anoxic paddy soil. ΔG was calculated from the data shown in Figs. 6 and 7 immediately before and about 4 days after addition of the exogenous electron acceptor. In fresh soil with added SO_4^{2-} H_2 eventually decreased to lower values, resulting in inhibition of CH_4 production

Experiment	H_2 (Pa)	ΔG (kJ mol^{-1} CH_4)	Inhibition of CH_4 production
Fresh paddy soil			
Control	5.7	−34.8	−
$+Fe^{3+}$	1.8	−18.0	−
$+SO_4^{2-}$	1.5	−17.4	−
Depleted paddy soil			
Control	3.1	−31.6	−
$+Fe^{3+}$	0.4	−8.0	+
$+SO_4^{2-}$	0.8	−11.0	+

Fig. 6 Effect of the addition of Fe^{3+} (30 m*M*) on the production of CH_4 and the partial pressure of H_2 in fresh (**a**) or depleted (**b**) anoxic paddy soil. In the depleted soil the final measurement was on day 60, for which the *symbols* are beyond the *frame of the figure. Closed symbols* control, *open symbols* plus ferrihydrite; ▽▼ H_2, ○● CH_4



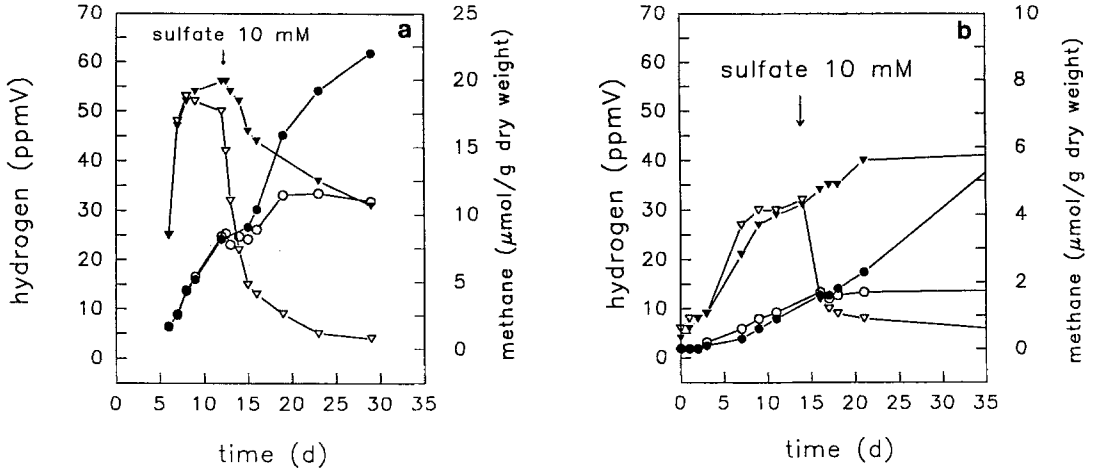


Fig. 7 Effect of the addition of SO_4^{2-} (10 m*M*) on the production of CH_4 and the partial pressure of H_2 in fresh (**a**) or depleted (**b**) anoxic paddy soil. For further explanations, see Fig. 6

tion from H_2 eventually became impossible when the ΔG had increased to values higher than -17 kJ mol^{-1} CH_4.

Discussion

Our experiments showed that the addition of an exogenous electron acceptor to anoxic paddy soil generally inhibited the reduction of electron acceptors with a lower standard redox potential, but only if the concentrations of electron donors were limiting in the soil. The addition of a suitable exogenous electron donor relieved the inhibition. These results are consistent with the concept that the dominance of a particular reduction process is due to the successful competition for common electron donors by those bacteria able to use the electron acceptor with the higher standard redox potential. Thus, such bacteria are able to deplete the common electron donor to a lower threshold concentration than the unsuccessful competitor.

Hence, the addition of NO_3^- slightly inhibited the reduction of Fe^{3+} and strongly inhibited the reduction of SO_4^{2-}, and to a larger extent in depleted than in fresh paddy soil. This observation indicates that the fresh paddy soil probably still had sufficient electron donors which could be used by the competing microorganisms. Reduction of SO_4^{2-} was reestablished only by the addition of exogenous H_2, and reduction of Fe^{3+} was stimulated by the addition of H_2. Obviously, the bacteria reducing NO_3^- and SO_4^{2-} had the potential to compete for H_2, and the bacteria reducing Fe_3^+ were stimulated by H_2. This result is reasonable in the light of our knowledge of these bacterial groups. NO_3^- reducers are known to use many different electron donors, including H_2, carbohydrates, acetate, and other fatty acids as well as more complex substrates such as peptone (Beauchamp et al. 1989). SO_4^{2-}-reducing bacteria, however, use a smaller number of electron donors. Enumeration of SO_4^{2-}-reducing bacteria in the Italian paddy soil showed that H_2-using SO_4^{2-} reducers were more numerous than those using acetate, lactate, or propionate (T. Wind and R. Conrad, unpublished data). Among the ferric iron reducers, *Shewanella (Alteromonas) putrefaciens* is known to use H_2 (Lovley et al. 1989). Enrichment cultures from the Italian paddy soil demonstrated the existence of Fe^{3+}-reducing bacteria which were able to use H_2, acetate, propionate, or benzoate as an electron donor (C. Achtnich and F. Bak, unpublished data).

Inhibition of the reduction of Fe^{3+} by NO_3^- has been demonstrated previously in marine sediments (Soerensen 1982) and in enrichment cultures from estuarine sediments (Tugel et al. 1986). This inhibition was interpreted as chemical reoxidation of Fe^{2+} to Fe^{3+} by NO_2^- produced during reduction of NO_3^- (Komatsu et al. 1978). However, accumulation of NO_2^- has never been observed in the NO_3^--treated Italian paddy soil. A biological oxidation of Fe^{2+} to Fe^{3+} by NO_3^--reducing bacteria would also be thermodynamically feasible, but microorganisms with these properties have not, to our knowledge, so far been described. Inhibition of reduction of Fe^{3+} by NO_3^- has also been demonstrated in heterotrophic soil bacteria that ferment glucose and have an NO_3^- reductase, i.e. *Bacillus polymyxa*, indicating that Fe^{3+} is reduced by the bacterial NO_3^- reductase system (Ottow 1970; Munch and Ottow 1977). Other fermenting bacteria, i.e., *Clostridium butyricum*, that do not have an NO_3^- reductase, and consequently do not reduce NO_3^-, reduce Fe^{3+} by another system that is not inhibited by NO_3^- (Munch and Ottow 1977). However, in these fermenting bacteria reduction of Fe^{3+} is only of minor importance for the generation of energy and consequently is considered to be a side reaction (Lovley 1991). Hence, the contribution of these fermenting bacteria to the reduction of Fe^{3+} in soil is probably small compared to those using Fe^{3+} reduction as an energy-generating mechanism such as *S. putrefaciens* (Lovley et al. 1989) or *Geobacter metallireducens* (Lovley et al. 1993).

Reduction of SO_4^{2-} was not only inhibited by the addition of NO_3^- but also by the addition of Fe^{3+} to

depleted paddy soil. In this case, the inhibition was relieved by the addition of H_2, acetate, or a mixture of potential electron donors to the paddy soil. However, H_2 was the most effective exogenous electron donor. This observation is consistent with the interpretation that H_2 was used by both the ferric iron and the SO_4^{2-} reducers, whereas acetate and fatty acids were mainly used by the ferric iron reducers. *S. putrefaciens* is known to use H_2 (Lovley et al. 1989), and *G. metallireducens* to use acetate, other short-chain fatty acids, and aromatics (Lovley et al. 1993). It was interesting that Fe^{3+} inhibited the reduction of SO_4^{2-} completely when it was added as additional exogenous (amorphous) Fe^{3+} (Fig. 4b), but that both electron acceptors were reduced simultaneously when Fe^{3+} was present as endogenous ferric iron (Fig. 1). Two explanations are conceivable: (1) The ratio of Fe^{3+} to SO_4^{2-} in relation to the available electron donors must be high enough to result in inhibition of SO_4^{2-} reduction, and this ratio is only reached by additional exogenous Fe^{3+}. (2) Endogenous Fe^{3+}, being at least partially crystalline, is less effectively reduced than amorphous exogenous ferrihydrite (Munch and Ottow 1980, 1982; Fischer 1983; Lovley 1991).

Production of CH_4 was inhibited by all of the other electron acceptors, i.e., NO_3^-, Fe_3^+, and SO_4^{2-}, when they were added to actively CH_4-producing paddy soil. Again, the inhibition was more pronounced in paddy soil which had previously been depleted in electron donors. In each case, however, the H_2 concentration in the anoxic paddy soil decreased when the exogenous electron donor was added. Obviously, H_2 was used to a lower concentration when an electron acceptor with a higher redox potential (e.g., SO_4^{2-}) became available to the soil microbial community. This result is consistent with the competition concept that microorganisms have a lower threshold for H_2 if they use an electron acceptor with a higher redox potential (Lovley and Goodwin 1988; Cord-Ruwisch et al. 1988).

The measurement of the H_2 concentrations allowed the calculation of the actual Gibbs free energies available for H_2-dependent methanogenesis before and after the addition of the exogenous electron acceptor. Before addition, the ΔG were lower than -31 kJ mol^{-1} CH_4, a value which is typical of anoxic paddy soil and other methanogenic environments (Conrad et al. 1986), and of methanogenic bacterial cultures (Seitz et al. 1990). After addition of Fe^{3+} or SO_4^{2-}, the ΔG in depleted soil increased to values higher than -17 kJ mol^{-1} CH_4, and methanogenesis eventually became inhibited. Obviously, the available energy was no longer sufficient to sustain methanogenesis. Moreover, this level of free energy is equivalent to less than 1/3 ATP, assuming that the irreversible synthesis of 1 mol ATP requires -70 kJ (Schink 1992), and 1/3 ATP seems to be the minimum amount of energy that can be harvested by a microorganism (Thauer and Morris 1984).

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